Biochem. J. (1981) **194**, 829–838
Printed in Great Britain

Factors controlling the expressed activity of histidine ammonia-lyase in the epidermis and the resulting accumulation of urocanic acid

Ian R. SCOTT
Unilever Research, Colworth Laboratory, Colworth House, Sharnbrook, Bedford MK44 1LQ, U.K.

(*Received 18 August 1980/Accepted 21 November 1980*)

The synthesis of urocanic acid by histidine ammonia-lyase in guinea-pig epidermis was investigated in various ways. 1. In epidermal homogenates the enzyme obeys Michaelis–Menten kinetics and shows marked dependence of its activity on pH, such that below pH 6 it is inactive. 2. Part-thickness skin samples cultured with radioactive histidine do not accumulate detectable radioactive urocanic acid during 3 days in culture. 3. Very little histidine ammonia-lyase activity can be detected in the living cells of the epidermis. The enzyme is almost completely restricted to the dead cells of the stratum corneum. 4. Radioactive histidine injected into living animals does not result immediately in the accumulation of radioactive urocanic acid. By 3 days after the injection, however, both radioactive urocanic acid and histidine appear, apparently at the expense of radioactive proteins. 5. In isolated stratum corneum, the residual histidine can be converted into urocanic acid by the histidine ammonia-lyase in the tissue only if the natural acidity of the tissue is neutralized. It is concluded from these observations that the biosynthesis of urocanic acid occurs naturally only in the stratum corneum, which contains only dead cells. The amount of urocanic acid accumulated is limited by the availability of free histidine produced by proteolysis of residual protein in these cells and by the penetration into the stratum corneum of the 'acid mantle' of the skin.

Urocanic acid is formed in a single-step reaction from L-histidine catalysed by the enzyme histidase (histidine ammonia-lyase, EC 4.3.1.3). The properties of this enzyme have been extensively studied in bacteria (Magasanik *et al.*, 1971; Rechler & Tabor, 1971; Klee, 1971), but rather less is known about the mammalian enzyme. Histidase occurs in large amounts in only two mammalian tissues, liver and epidermis (Zannoni & La Du, 1963). In liver it is present as the first enzyme of the major catabolic route of histidine metabolism, and the resulting urocanic acid is further metabolized in a series of reactions culminating in complete degradation to CO_2 and water. Absence of this enzyme results in the disorder known as histidinaemia (La Du, 1972). In the epidermis the other enzymes of this catabolic pathway are absent (Zannoni & La Du, 1963; Baden & Pathak, 1967), and as a result urocanic acid accumulates, forming as much as 0.5% of the dry weight of the epidermis (Tabachnik, 1959). The function of this accumulation of urocanic acid has not been established, although the fact that it absorbs u.v. light in the erythemal part of the spectrum has led to the widespread belief that it acts as a natural sunscreen.

Little is known about the control of the activity of the enzyme *in vivo*. The total amount of enzyme in liver and epidermis is under differential hormonal control (Feigelson *et al.*, 1976), but the means of regulation of expressed activity *in vivo* is unknown. It has been suggested that the relatively high K_m of the hepatic enzyme at neutral pH results in control of activity by substrate limitation, thus preventing the unnecessary loss of irreplaceable histidine while allowing catabolism of excess dietary amino acid (Brand & Harper, 1976). Reported values for the K_m of the epidermal enzyme are, however, much lower (Baden & Gavioli, 1974), suggesting that some other control factor acts in the epidermis to prevent excessive histidine degradation.

Phosphorylation of the enzyme has been proposed as a method of control of activity (Nikolaev *et al.*, 1977), but the significance of this is doubtful, as the specific activity of the enzyme, from both sources, is constant at all stages of development (Feigelson *et al.*, 1976).

In the epidermis, changes in the amount of urocanic acid have been reported in response to a variety of factors, including the area of the body studied, the ethnic origin of the subject (Kral *et al.*, 1967, 1968), the extent of exposure to u.v. light (Everett *et al.*, 1961; Hais & Strych, 1968; Wadia

0306-3283/81/030829-10$01.50/1

et al., 1975) and to nicotinic acid therapy (Vasantha, 1970). Changes in histidase activity have also been reported, but no clear correlation between histidase activity and urocanic acid content has been established. Very little, therefore, is known about the factors responsible for the control of the activity of histidase in the epidermis or for the resulting accumulation of urocanic acid. The aim of the work described here was to establish the principal factors controlling the accumulation.

Experimental

Materials

Male albino guinea pigs (400g body wt.) of the Dunkin Hartley strain (Colworth sub-strain) were used throughout this study. L-[2,5-^{3}H]Histidine was obtained from The Radiochemical Centre (Amersham, Bucks., U.K.) as a solution in 2% ethanol, of specific radioactivity 40–60Ci/mmol. It was used for injections as supplied. Culture media and antibiotics were obtained from Gibco Bio-Cult. Dispase (grade II) was obtained from Boehringer Corp. (London) Ltd., urocanic acid was from Koch–Light Laboratories, histidine and NADH were from Sigma, diphenylamine was from Serva, ion-exchange resins were from Bio-Rad Laboratories, materials for scintillation counting and Soluene^{350} were from Packard. All other chemicals were of analytical-reagent grade.

Determination of the kinetic constants of epidermal histidase

The dorsal skin of a guinea pig was shaved closely, excised and placed between two glass plates. The glass plates were placed on a smooth block of solid CO_2 so that the dermal side of the skin faced the block and the skin was allowed to freeze. The upper glass plate was then removed and the epidermis scraped from the skin with a scalpel. This procedure, freeze-scraping, results in a preparation of interfollicular epidermis with very little dermal contamination (W. E. Sprott, personal communication) and minimizes the possibility of damage to the epidermis by heat or chemicals used in other methods of epidermal isolation.

The frozen epidermis was homogenized in the proportions of 10cm^2 to 1ml of 50mM-Na_2HPO_4/KH_2PO_4, pH8.0, in a glass/glass homogenizer at 0–4°C. The homogenate was centrifuged (1500*g*, 5min, 4°C) and the supernatant dialysed overnight at 0–4°C against 100vol. of 1mM-Na_2HPO_4/KH_2PO_4 (pH8.0)/0.9% NaCl/50μM-zinc acetate. The resulting precipitate was removed by centrifugation (1500*g*, 5min, 4°C) and the supernatant frozen in batches and later used for enzyme assays.

Histidase was assayed by monitoring, for at least 10min, the increase in A_{277} caused by the production of urocanic acid. The assay contained: 1.5ml of 0.2M-Na_2PO_4/KH_2PO_4 of the appropriate pH; 1.0ml of 1.8% NaCl/0.1mM-zinc acetate, and enzyme plus 0.9% NaCl to 3.0ml final volume. The mixture was incubated at 37°C and the reaction started by the addition of L-histidine. No reaction occurred in the absence of either enzyme or histidine and the reaction was linear with time up to an A_{277} of 1.5. This A_{277} was not exceeded during the period of the assay. The kinetic data were processed graphically by the method of Eisenthal & Cornish-Bowden (1974).

Separation of histidine and urocanic acid from epidermal homogenates

Epidermis was homogenized in 0.6M-$HClO_4$ and the supernatant neutralized with KOH and Tris/HCl buffer to give a final concentration of 50mM-Tris, pH6–8. The neutralized sample was chilled and the $KClO_4$ removed by centrifugation (1500*g*, 5min, 4°C). A 1.0ml portion of the supernatant was applied to a column (0.7cm × 2.0cm) of AG 1 X 8 (200–400 mesh) ion-exchange resin equilibrated with 0.1M-Tris/HCl, pH8.1. The sample was eluted with a total of 3.0ml of the same buffer, which recovered more than 95% of applied radioactive histidine. The column was then washed twice with 10ml of the same buffer, followed by 5ml of water, and the urocanic acid was eluted with 3.0ml of 0.1M-HCl. Urocanic acid standards, assayed by the A_{266}, were recovered quantitatively (98 ± 1%).

Preparation and assay of part-thickness skin samples

Part-thickness skin samples were obtained from the shaved skin of guinea pigs with a Castroviejo keratotome set at a nominal thickness of 0.2mm. This produced skin samples consisting of epidermis supported on a thin layer of dermis containing no sebaceous glands. To obtain samples of precisely defined area, the skin was cut part way through with a circular skin-biopsy punch before use of the keratotome. When skin samples were to be maintained in culture, the skin was first washed thoroughly with 70% (v/v) ethanol and the keratotome and blade were sterilized by autoclaving.

To determine the histidase and urocanic acid contents of such samples, they were homogenized at 0–4°C in 1.0ml of PBS medium (0.8% NaCl, 0.02% KCl, 0.02% KH_2PO_4, 0.14% Na_2HPO_4,2H_2O) containing 33μM-zinc acetate. Then 0.2ml of 5mM-L-histidine containing 5μCi of L-[2,5-^{3}H]histidine/ml was added to 0.8ml of the homogenate and the mixture was incubated at 37°C for 1h. The reaction was stopped by chilling and the addition of 0.2ml of 2.4M-$HClO_4$, and the resulting precipitate was removed by centrifugation (1500*g*, 5min, 4°C). The supernatant was neutralized and urocanic acid

isolated as described above. Urocanic acid was estimated by its A_{266} (in acid solution), and the histidase activity was calculated from the amount of [^{3}H]urocanic acid produced in the 1h incubation. Under these conditions the reaction proceeded at a uniform rate for at least 2h.

Culture of part-thickness skin samples

Sterile 6mm-diameter part-thickness skin samples prepared as described above were floated on Minimum Essential Medium (Earle's salts) containing 100i.u. of penicillin and streptomycin/ml and 2.5μg of Fungizone/ml. They were incubated at 37°C in an atmosphere of air/CO_2 (19:1) in vented trays. The medium was replaced daily. To measure the histidase activity 1μCi of L-[2,5-^{3}H]histidine/ml was added to the medium. Used medium was acidified to 0.4M-$HClO_4$, any precipitate was removed by centrifugation (1500*g*, 5min, 4°C), the supernatant was neutralized and the [^{3}H]urocanic acid isolated as described above. The skin samples were washed briefly in PBS medium and homogenized in 1.0ml of 0.4M-$HClO_4$. The radioactive protein precipitate was washed in fresh 0.4M-$HClO_4$, dissolved in 1.0ml of Soluene^{350} and counted for radioactivity in toluene/0.5% 2,5-diphenyloxazole/0.02% 1,4-bis-(5-phenyloxazol-2-yl)benzene. The $HClO_4$-soluble fractions was neutralized and [^{3}H]urocanic acid isolated as described above.

Samples cultured for various times were fixed in Bouin's fluid, embedded in paraffin, sectioned and stained with haematoxylin and eosin by standard procedures.

Fate of [^{3}H]histidine injected in vivo

[^{3}H]Histidine (40μCi) was injected intradermally into guinea pigs under light cyclopropane anaesthesia by using a PB600 repeating dispenser with a 1.0ml gas-tight syringe (Hamilton). The hypodermic needle was passed completely through the skin, traversed subcutaneously for approx. 2cm and inserted into the dermis from below. The injected histidine (40μl) raised a bump, which persisted for a few minutes and was marked. This procedure was used to avoid damage to the epidermis at the point of injection of the radioisotope.

Epidermis was isolated by freeze-scraping from a disc of skin of 1.3cm diameter taken from the injection site after the appropriate time. It was homogenized in 0.4M-$HClO_4$, and protein, urocanic acid and histidine were separated as described above. The radioactivity of the protein was measured directly, but the eluates from the ion-exchange column, containing histidine and urocanic acid, were tested for radiochemical purity by isotope-dilution analysis. To the two 3ml column eluates were added, respectively, 60mg of histidine and 30mg of anhydrous twice-recrystallized urocanic acid. The concentrations of histidine and urocanic acid were measured in arbitrary units by the absorbance at 210 and 277nm respectively of 10^3-fold dilutions of the samples in 40mM-HCl and 32mM-Tris/HCl, pH8.1, respectively. The radioactivity of 1.0ml samples was determined by counting in MI97 scintillator.

The histidine and urocanic acid were then crystallized, the histidine by the addition of 5vol. of A.R. acetone and chilling, the urocanic acid by neutralization with 1M-NaOH and chilling. The crystals were collected, dried and redissolved in 2.0ml of water. The specific radioactivity was determined as described above, and from the decrease in the specific radioactivity the radiochemical purity of the original samples was determined.

Location of histidase after separation of different epidermal cell types

Discs of part-thickness skin of 1.3cm diameter were floated on 1.0ml of PBS medium containing 100i.u. of penicillin and streptomycin/ml and 0.25% Dispase for 1h at 37°C. The epidermis could then easily be peeled away from the dermis. The epidermis was incubated in 1.0ml of fresh medium containing Dispase and antibiotics for 3h at 37°C with occasional, very gentle shaking. This procedure was monitored histologically and was shown to remove all nucleated cells from the epidermis, leaving the stratum corneum intact. The cells were centrifuged (400*g*, 5min, 4°C) and washed in cold PBS medium. The stratum corneum was washed manually in cold PBS medium. The dermis, nucleated epidermal cells and stratum corneum were all separately homogenized in 1.2ml of PBS medium containing 33μM-zinc acetate and 0.1% Triton X-100. The two 1.0ml solutions of Dispase were collected, chilled, 0.2ml of 2.4M-$HClO_4$ was added and the precipitate collected.

Two 0.2ml samples of the homogenized dermis, cells and stratum corneum were assayed for histidase activity by addition of 0.5ml of 0.2M-Na_2HPO_4/KH_2PO_4, pH7.4, 0.2ml of water and 0.1ml of 10mM-L-histidine containing 10μCi of [^{3}H]histidine/ml. After 1h at 37°C the reaction was stopped by chilling and the addition of 0.2ml of 2.4M-$HClO_4$, and the [^{3}H]urocanic acid was isolated as described above. The presence of Triton X-100 was shown not to affect the assay.

A further sample of the homogenate was centrifuged (1500*g*, 5min, 4°C) and the supernatant assayed spectrophotometrically for lactate dehydrogenase at 37°C in a mixture containing 50mM-potassium phosphate, pH7.6, 0.3mM-sodium pyruvate and 0.1mM-NADH (Bergmeyer *et al.*, 1963). A 0.5ml sample of the homogenate was chilled and

acidified with 0.2ml of 2.4M-$HClO_4$ and the precipitate collected. Both this precipitate and those from the two solutions of Dispase were digested in 1.5M-$HClO_4$ at 70°C for 30min, centrifuged (1500*g*, 5min, 4°C), and the supernatant was assayed for DNA by the method of Giles & Myers (1965).

Untreated part-thickness skin samples were homogenized directly and assayed in the same way as the isolated components.

Measurement of histidine and urocanic acid content of isolated stratum corneum

Samples of stratum corneum prepared by the use of Dispase were homogenized in 0.4M-$HClO_4$ at 4°C, and then centrifuged (1500*g*, 5min, 4°C). The supernatant was neutralized by the addition of KOH and Tris/HCl buffer, pH8.1, to a final concentration of 80mM-Tris. The $KClO_4$ was allowed to precipitate at 0°C, centrifuged (1500*g*, 5min, 4°C), and the supernatant removed. The urocanic acid concentration was determined by measuring the A_{277} of an appropriate dilution of the supernatant, and the histidine concentration by the fluorimetric method of Hakanson *et al.* (1974), with the modification that 0.1M-Na_2CO_3/$NaHCO_3$, pH10.3, was used as buffer instead of 3mM-NaOH. Standard L-histidine was added to duplicate samples as an internal standard to correct for quenching of the fluorescence by the sample. The molar ratio of urocanic acid to histidine was calculated and the percentage of the residual histidine that had been converted into urocanic acid during an incubation was calculated from the equation

$$\%\ \text{converted} = \left[1-\frac{(1+x)}{(1+y)}\right] \times 100$$

where x is the mean ratio urocanic acid/histidine before the incubation and y is the ratio after the incubation.

Results and discussion

Kinetic constants of epidermal histidase

Figs. 1(*a*) and 1(*b*) show that in the guinea pig both the K_m and $V_{max.}$ of the histidase preparation are pH-dependent. The K_m varies between 0.23mM and 0.4mM over the range pH6.2–8.3, with a minimum at pH7.1. These values are considerably lower than those for the rat liver enzyme, whose K_m varies from over 2.6mM to 1.0mM over the same pH range (Brand & Harper, 1976), and which has a minimum K_m of 0.45mM at pH9.0.

Differences in the K_m of histidase from liver and epidermis have been reported (Baden & Gavioli, 1974), but their assays were performed at alkaline pH and therefore give little indication of the likely activity of the enzyme *in vivo*.

The $V_{max.}$ is markedly pH-dependent and the enzyme is almost inactive at pH values below 6.5. Although there is no information available about intracellular histidine concentration and pH value in the epidermis, the mean histidine concentration in whole guinea-pig epidermis can be calculated to be of the order of 10mM (Tabachnik, 1959), so it is unlikely that the histidase activity is limited by histidine concentration, as could be the case for the liver enzyme, with its higher K_m.

There is no reason to believe that the intracellular pH of the nucleated epidermal cells (those still active in macromolecular synthesis, as distinct from the dead cornified cells of the stratum corneum) differs

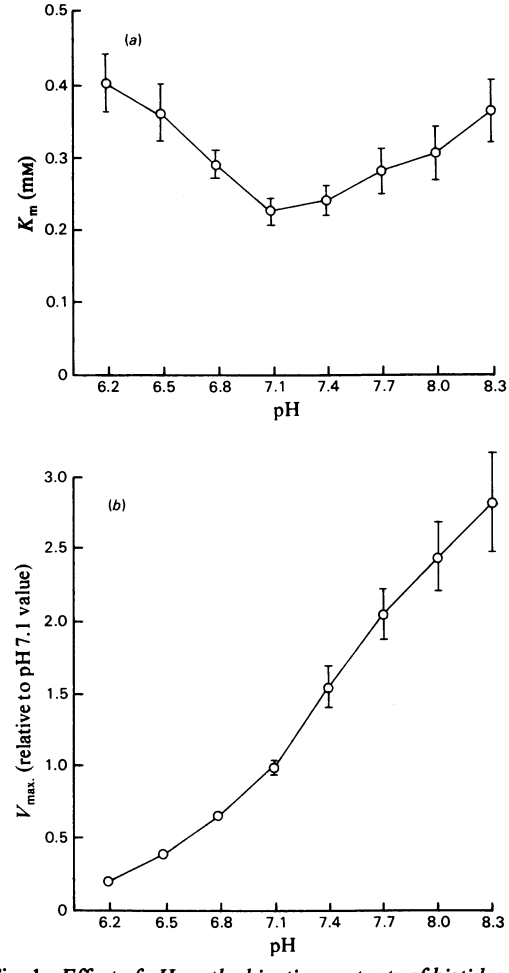

Fig. 1. *Effect of pH on the kinetic constants of histidase*
The kinetic constants (*a*, K_m; *b*, $V_{max.}$) of a partially purified preparation of epidermal histidase were determined as described in the Experimental section. The data are presented as means ± S.D. for at least five determinations on two separate preparations of enzyme. Where no S.D. is shown, the S.D. was smaller than the size of the symbol.

from the normal physiological values. Therefore the activity of histidase in those cells, though not maximal, would be expected to be between one-third and one-half of the maximum value (Fig. 1). It should be noted, however, that the surface of the skin is relatively more acidic (Marples, 1965) and that this 'acid mantle' must penetrate some depth into the stratum corneum. Under such conditions (pH <6) histidase activity would virtually cease (see below).

Under what might be expected to be likely physiological conditions (pH 7.4, 1 mM-histidine), the activity of histidase in 6 mm-diameter 0.2 mm-thick part-thickness skin samples was found to be 282 ± 67 nmol/h per cm^2 of epidermis, and the urocanic acid content was 368 ± 74 nmol/cm^2 of epidermis (mean $\pm$ S.D. for 55 samples from four animals). There was very little correlation between histidase activity and urocanic acid content (correlation coefficient 0.41). In view of this very high activity of histidase, relative to the concentration of urocanic acid ultimately produced, it is unlikely that the available enzyme is the limiting factor controlling the concentration of urocanic acid accumulated by the epidermis.

Expression of histidase activity by cultured part-thickness skin samples

Skin samples (0.2 mm thick) floating on suitable media (see the Experimental section) maintain viability, measured by nucleic acid and lipid synthesis, for up to 24 h and continue many metabolic functions, including protein synthesis and glucose metabolism, for longer periods (Prottey *et al.*, 1972; Bailey, 1971; P. J. Hartop, personal communication). Histologically, the skin appears fairly normal up to 3 days, but then deteriorates, with oedema and necrosis in the outer layers and almost complete loss of the granular layer of the epidermis.

Incubation of skin samples in medium containing radioactive histidine for up to 5 h resulted in a constant rate of formation of radioactive urocanic acid for at least 5 h. The rate of synthesis was approximately one-tenth of that expected for the homogenized tissue. Kinetic studies showed that the K_m for histidine was exactly that expected for the homogenized tissue (results not shown), with no indication of a component of the activity with a lower K_m, as would be expected for an intracellular enzyme. Further, it was found that all the synthesized urocanic acid was present in the medium rather than the tissue and that most of the detected histidase activity was also present in the medium in a soluble form. Thus most of the histidase activity detected resulted from enzyme exposed to the medium by damage, probably physical, of the epidermis, and in the intact tissue the activity of the histidase to exogenous histidine was very low.

On the assumption that the intact cells of the epidermis might be synthesizing and accumulating urocanic acid at a very low rate, commensurate with the final concentration of urocanic acid accumulated, skin samples were incubated in medium containing radioactive histidine for longer periods, and the concentrations of radioactive urocanic acid and protein in the samples measured (Fig. 2). For 3 days the concentration of radioactive urocanic acid remained constant at a background value (possibly resulting from urocanic acid absorbed from the culture medium) while synthesis of radioactive proteins continued at a constant rate. Between 3 and 4 days the concentration of radioactive proteins stabilized and a small amount of radioactive urocanic acid appeared. This finding cannot be interpreted with confidence, as after 4 days in culture the tissue had deteriorated considerably. It does, however, suggest that one route of urocanic acid synthesis may be via breakdown of previously synthesized proteins, which would explain the apparent inability of epidermal histidase to act on exogenous histidine. This possibility prompted investigations *in vivo*, where longer time courses could be studied.

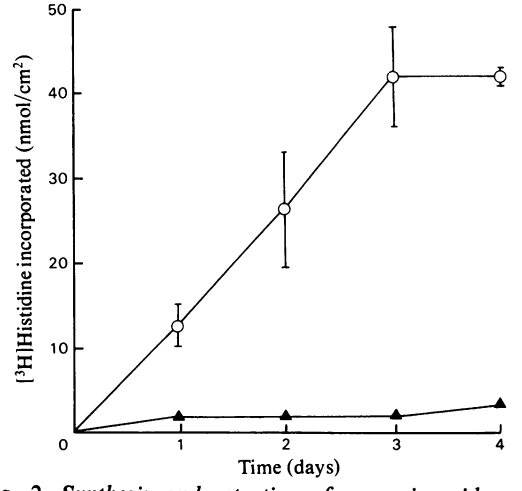

Fig. 2. *Synthesis and retention of urocanic acid and protein by epidermis in culture*

Part-thickness skin samples (6 mm diameter) were floated on culture medium containing [^{3}H]histidine as described in the Experimental section. After the stated period the skin sample was removed, washed and the radioactivity present as protein (O) and urocanic acid (▲) determined. The specific radioactivity of histidine in the medium was calculated from the stated concentration of histidine in the medium and was assumed to be unaffected by small amounts of histidine in the tissue. Where no error bar is shown, the S.D. was smaller than the symbol.

Synthesis of urocanic acid in vivo

[^{3}H]Histidine was injected intradermally, rather than subcutaneously or intraperitoneally, so that the radiolabelling pattern approximated to pulse–chase kinetics. This is shown in Fig. 3, which shows that the free radioactive histidine content of the epidermis at the site of injection remained high for only a few minutes, corresponding to the lifetime of the fluid-filled intradermal bump produced by the injection. Thereafter, the very-high-specific-radioactivity histidine was diluted and displaced by non-radioactive histidine. The resulting systemic concentration of [^{3}H]histidine resulted in the uptake by the epidermis of less than 1% as much radioactivity as the immediate injection. It was therefore possible to give a single animal multiple injections at intervals and hence follow the fate of the incorporated radioactivity as a function of time.

Fig. 3 shows that the concentration of radioactive histidine fell rapidly after injection, as the radioactive histidine was both displaced from the tissue and rapidly incorporated into protein, which after a few hours accounted for almost all the radioactivity. Even when the radioactivity of histidine was high, however, virtually no radioactive urocanic acid was detected, confirming that the epidermal histidase does not act directly on exogenous histidine. The concentrations of [^{3}H]histidine and [^{3}H]urocanic acid remained very low for 2 days, but then rose, apparently at the expense of ^{3}H-labelled protein, until the final distribution of radioactivity was approx. 17% histidine, 33% protein and 50% urocanic acid. This ratio remained stable for at least 20 days. Isotope-dilution analysis of the histidine- and urocanic acid-containing fractions separated by ion-exchange chromatography showed that the two compounds accounted for essentially all the soluble radioactivity for 4 to 26 days (purity of urocanic acid $96 \pm 4\%$, purity of histidine $98 \pm 6\%$), but that in the first few hours after injection the urocanic acid fraction contained as much as 70% radioactivity that was not due to urocanic acid. This presumably reflects the action of alternative pathways of histidine catabolism in the epidermis, resulting in compounds such as imidazolylacetic acid, which would co-chromatograph with urocanic acid in the ion-exchange system used. No such alternative pathways were evident after 2 days, however.

From these results it appeared that the epidermal histidase was present in a compartment of the epidermis not accessible to exogenous histidine and whose only source of histidine was via protein degradation. Two possible methods of such compartmentation are that the histidase is in a separate intracellular compartment or that it is restricted to a particular class of cells. The second possibility seems more likely, particularly in view of the long delay between synthesis of the protein and its breakdown and conversion into urocanic acid. However, radioautographic studies have shown that all nucleated cells of the epidermis are accessible to exogenous histidine (Fukuyama & Epstein, 1966), so if this hypothesis is correct, the histidase must be restricted to the dead, fully differentiated cells of the stratum corneum. This possibility was tested by direct measurement of histidase activity in the nucleated cells and the stratum corneum.

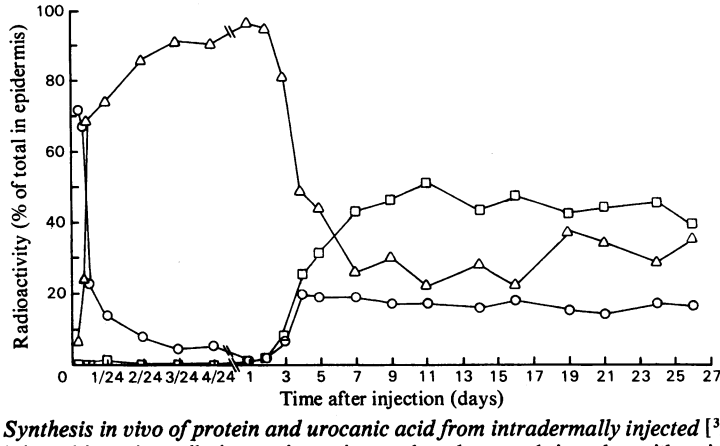

Fig. 3. *Synthesis in vivo of protein and urocanic acid from intradermally injected [^{3}H]histidine*

[^{3}H]Histidine was injected intradermally into guinea pigs, and at the stated time the epidermis was isolated from the injection site by freeze-scraping. The protein (△), urocanic acid (□) and histidine (○) were separated as described in the Experimental section and the radioactivity of each fraction was expressed as a percentage of the total radioactivity in the epidermal sample.

Location of histidase by enzymic separation of the cells of the epidermis

Techniques for the isolation of epidermal cells by controlled digestion of the epidermis with trypsin are well established (Prottey *et al.*, 1974). However, cells prepared by this technique from the guinea-pig epidermis used in this study were found to have low viability (50–75%), as measured by Trypan Blue dye exclusion. Because it was observed that the more differentiated cells of the epidermis tended to have particularly low viability, it was feared that selective loss of such cells could occur during separation with trypsin. Such losses could severely distort the true distribution of histidase. A superior method of cell separation was therefore sought and found in the enzyme Dispase, which has been used very successfully in culture of cells from a variety of tissues (Matsumura *et al.*, 1975). The action of this proteinase is so mild that cells can be grown in the presence of the enzyme for long periods without loss of viability.

Cells isolated from guinea-pig epidermis with the enzyme, as described in the Experimental section, had extremely high viability ($97.3 \pm 1.1\%$) as shown by Trypan Blue dye exclusion, and maintained the same viability after several hours exposure to the enzyme. The enzyme removed all nucleated cells from the epidermis, as demonstrated histologically, leaving an apparently undamaged stratum corneum. The distribution of histidase was compared with that of DNA, a marker of all nucleated cells whether viable or lysed, and lactate dehydrogenase, which, because it was rapidly degraded by Dispase, was a marker of intact cells only. Table 1 shows the recovery of these materials during the isolation of cells with Dispase. Small losses of histidase occurred, which approximated to the proportion of histidase released from the tissue by physical damage during the cutting of the skin with the keratotome (see above). Rather larger losses of lactate dehydrogenase occurred, indicating that some cell damage was still taking place, despite the high viability of the surviving cells. As the degree of cell damage, measured by lactate dehydrogenase destruction, was appreciably greater with trypsin, Dispase was used in subsequent experiments, although the possibility of selective loss of some cell types must be considered in the interpretation of the results.

Table 2 shows the distribution of histidase, lactate dehydrogenase and DNA among the various fractions. The loss of 8% of the total DNA into the Dispase solution used to separate the epidermis and dermis probably represents destruction of dermal and follicular cells, as the interfollicular epidermis at this stage showed no histologically detectable damage. The loss of a further 16% of the total DNA on disaggregation of the cells of the epidermis confirms that some loss of epidermal cells does take place during this procedure.

The absence of DNA from the stratum corneum confirms the histological observation of the complete removal of nucleated cells, and the presence of 93% of the recovered histidase in the stratum corneum, in stark contrast with lactate dehydrogenase, confirms that the greatest part of the histidase is indeed present in this compartment of the epidermis.

Table 2. *Distribution of DNA, histidase and lactate dehydrogenase after fractionation of skin with Dispase*

Keratotome slices of 0.2 mm nominal thickness and 1.3 cm diameter were fractionated and assayed as described in the Experimental section. Dispase 1 refers to the enzyme used to separate the epidermis from the dermis, and Dispase 2 refers to that used to disaggregate the cells of the epidermis. Each result is the mean ± S.D. for four samples, and is expressed as a percentage of the total recovered (Table 1).

Tissue fraction	DNA	Histidase	Lactate dehydrogenase
Dermis	45 ± 2	0.5 ± 0.3	54 ± 4
Epidermal cells	31 ± 3	5.2 ± 1.2	44 ± 3
Stratum corneum	1.0 ± 0.2	93 ± 3	2.0 ± 0.6
Dispase 1	7.8 ± 1.8	0	0
Dispase 2	16 ± 1	0	0

Table 1. *Recovery of DNA, histidase and lactate dehydrogenase after fractionation of skin with Dispase*

Four keratotome slices of 0.2 mm nominal thickness and 1.3 cm diameter from guinea-pig skin were homogenized in 1.2 ml of PBS medium containing 33 μM-zinc acetate and 0.1% Triton X-100 and assayed as described in the Experimental section. Four other similar slices from the same animal were fractionated as shown in Table 2 and the amounts of each substance in all the fractions were totalled. The results show the means ± S.D. for the four samples.

	DNA ($\mu g/cm^2$)	Histidase (nmol/h per cm^2)	Lactate dehydrogenase (nmol/min per cm^2)
Total in unfractionated keratotome slice	81 ± 11	254 ± 10	528 ± 69
Total in fractionated keratotome slice	87 ± 13	208 ± 45	342 ± 62
Recovery (%)	108 ± 20	82 ± 15	65 ± 13

The histidase present in the nucleated-cell fraction of the epidermis is probably partly due to contamination of these cells with anucleate squames released from the upper surface of the stratum corneum during the treatment with Dispase. An upper limit can be placed on the possible histidase content of the nucleated cells from the data on the recovery of histidase, as, of the 18% lost during the fractionation, at least 10% can be accounted for by the histidase released by physical damage to the epidermis by the keratotome (see above).

Therefore, although these experiments demonstrate that most of the histidase is restricted to the stratum corneum, there may be a limited amount of histidase in the nucleated cells. If there is any such enzyme, however, it seems to be prevented from acting *in vivo*, as shown by the data in Fig. 3. Whether this is because of compartmentation within the cell, the existence of an inactive proenzyme or other factors such as competition for histidine by other reactions, has not been investigated.

Effect of pH on the histidase activity of the stratum corneum

Fig. 3 shows that conversion of histidine, derived apparently from proteolysis, into urocanic acid was not complete even after several weeks, despite the presence of high activities of histidase in the stratum corneum. Such incomplete conversion is not due to an equilibrium, as the action of histidase is irreversible under normal conditions. Possible explanations for this incomplete use of available substrate include separate compartmentation of enzyme and substrate, proteolytic destruction of the histidase in the upper layers of the stratum corneum or inhibition of the histidase activity in the upper layers of the stratum corneum. The first possibility was judged unlikely because of the lack of subcellular compartmentation in the cells of the stratum corneum, and the second because histidase activity is measured in the superficial layers of the stratum corneum as a diagnostic test for histidinaemia. The third possibility was, however, considered likely because of the known acidity of the upper layers of the stratum corneum (Marples, 1965) and the inhibition of the activity of epidermal histidase at such acid pH values (Fig. 1).

To test this possibility, samples (1 cm × 1 cm) of pure stratum corneum were prepared by using Dispase as described above. Varying quantities of histidine and urocanic acid were lost from the tissue during this procedure, but it was shown that this represented random loss rather than preferential loss of either newly synthesized or older urocanic acid (Fig. 4). The histidine and urocanic acid contents of such isolated samples of stratum corneum varied depending on the extent of the loss, but the ratio of the two remained quite constant for samples from a

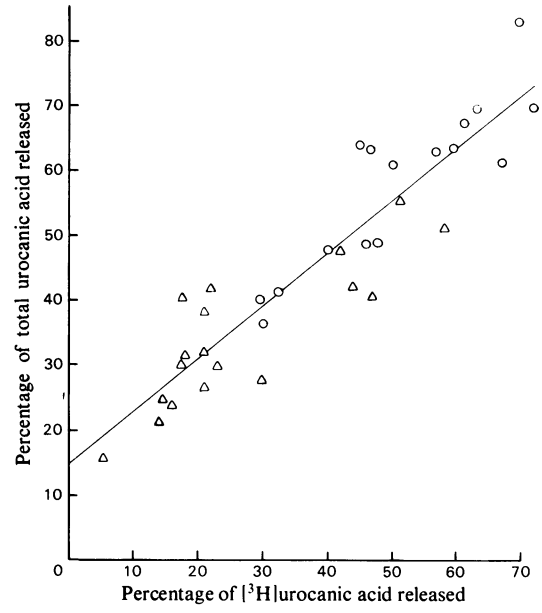

Fig. 4. *Comparison of the release of newly synthesized (^{3}H-labelled) and total urocanic acid from small and large part-thickness skin samples floating on PBS medium*

Part-thickness skin samples of 1.4 cm (△) and 0.6 cm (○) diameter were taken from sites on the skin of guinea pigs that had been injected intradermally with 40 μCi of [^{3}H]histidine 4 days earlier. The samples were floated on 1 ml of PBS medium at 37°C for 3 h and the total and ^{3}H-labelled urocanic acid contents of both tissue and medium determined.

given animal and was very similar to both the ratio for whole epidermis and the radioisotopic ratio found in Fig. 3. The histidine and urocanic acid present in these stratum-corneum samples were therefore representative of that present in the normal stratum corneum.

Samples of stratum corneum were incubated in a variety of buffers for 18 h and the molar ratio of urocanic acid to histidine was determined. From the change in this ratio after the incubation, the percentage of the histidine in the stratum-corneum sample that had been converted into urocanic acid during the incubation was determined. The results are shown in Fig. 5. Incubation in saline alone (*b*) caused very little conversion of histidine, but incubation in dilute NH_3 (*f*) or alkaline buffers containing NH_4Cl (*d* and *e*) caused up to 80% conversion. Thus the normal conversion *in vivo* of approx. 75% of the available free histidine in the stratum corneum into urocanic acid (Fig. 3) can be increased to 95% *in vitro* simply by exposing the stratum corneum to an alkaline solution containing

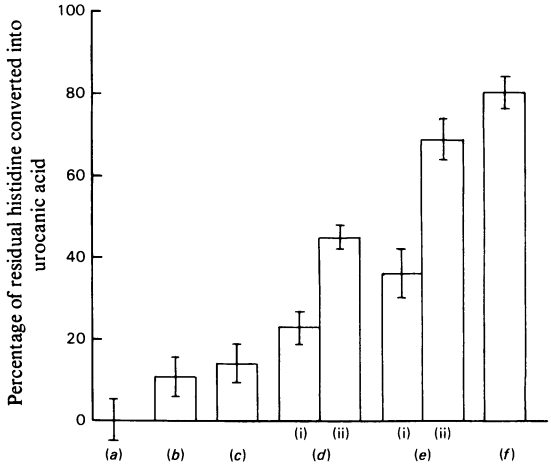

Fig. 5. *Conversion of residual histidine in the stratum corneum into urocanic acid after incubation in various media*

Samples of stratum corneum (1 cm × 1 cm) were prepared as described in the Experimental section, and incubated overnight at 37°C in various media: (*a*), not incubated; (*b*), 0.9% NaCl; (*c*), 10mM-NH_4Cl; (*d*), 90mM-Na_2HPO_4/10mM-NaH_2PO_4, pH 7.7, alone (i) or plus 10mM-NH_4Cl (ii); (*e*), 100mM-Tris/10mM-HCl, pH 9.0, alone (i) or plus 10mM-NH_4Cl (ii); (*f*), 10mM-NH_3. The stratum corneum was removed and the percentage of the original histidine in the stratum corneum that had been converted into urocanic acid during the incubation was determined as described in the Experimental section. Error bars denote S.D.

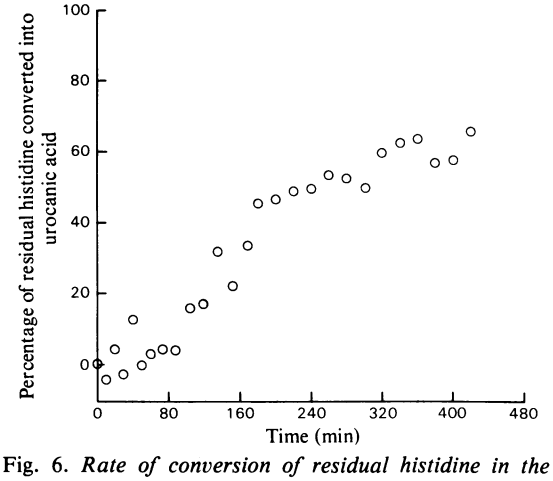

Fig. 6. *Rate of conversion of residual histidine in the stratum corneum into urocanic acid on incubation in 100mM-Tris/10mM-HCl, pH 9.0, containing 10mM-NH_4Cl*

Samples of stratum corneum were treated as described in the legend to Fig. 5, except that the incubation period was shortened to 0–420 min, as shown.

NH_4^+ ions. The lack of effect of unbuffered NH_4Cl solution (*c*) suggests that the active species is the uncharged NH_3 molecule, which is reasonable in view of the ability of this molecule to penetrate through lipid membranes, in contrast with the virtual impermeability of such membranes to hydrophilic molecules such as Tris and phosphate.

The action of the alkaline solutions on the stratum corneum was relatively rapid, as shown in Fig. 6. After a short lag period, probably representing the time required for penetration of the NH_3, the conversion of histidine proceeded at an approximately uniform rate for 2 h, followed by a much slower reaction of the remaining histidine. It is clear therefore that the incomplete utilization of the available histidine substrate is principally due to inactivation of the enzyme by the progressively decreasing pH as the cell moves close to the surface of the skin.

Conclusions

Guinea-pig epidermis consists of several layers of cells. A single layer of small, actively dividing, cells constitutes the basal layer. Above this are one or two layers of larger, non-dividing, cells, the spinous layer, and above these is a single layer of flattened but still metabolically active cells, the granular layer.

In the model proposed on the basis of the above results, histidase is synthesized in one or more of these layers of metabolically active cells, but for some reason does not act on histidine in these cells to any appreciable extent. Such a restriction on the activity of the enzyme may be essential in the epidermis, as the cells of the granular layer, furthest from the vascular system of the dermis, have a large requirement for histidine for the synthesis of certain histidine-rich proteins, which are a major component of these cells (Ball *et al.*, 1978).

The granular cells over a period of 24–48 h become metabolically inactive, break down most of their organelles and transform into anucleate squames, which form the stratum corneum. During this process most of the non-structural proteins of the cell are degraded, but the histidase enzyme is resistant and at this stage becomes active, although whether this is due to the breakdown of intracellular structure, activation of a proenzyme, destruction of an inhibitor or another mechanism is unknown. The active histidase acts on histidine produced by degradation of cell proteins, synthesizing urocanic acid, which, because the plasma membrane of the cell becomes highly impermeable on keratinization, is accumulated. The breakdown of proteins is eventually complete, but by this time the pH of the cell has fallen to the point at which the histidase is inactive, and so the remaining histidine pool is stable.

Five principal factors therefore control the final extent of accumulation of urocanic acid. Firstly, the total available substrate will be determined by the amount of protein in the cell available for breakdown. This will normally be broadly constant for a given species. Secondly, the substrate actually available when the histidase becomes active will depend on the extent to which the proteins of the cell are broken down and reabsorbed by the epidermis before the cell membrane becomes impermeable during keratinization. In Fig. 3, during the interval between 0 and 2 days after injection, when no radioactive histidine or urocanic acid can be detected in the epidermis, it is probable that a certain amount of proteolysis is taking place, but that the resulting radioactive histidine is rapidly lost from the epidermis or resynthesized into protein.

Thirdly, the completeness of the breakdown of available protein in the stratum corneum to amino acids will also affect the availability of histidine. Normally such breakdown is very efficient, leaving little residual soluble protein, but under abnormal conditions of rapid epidermal growth considerable quantities of soluble protein may persist in the stratum corneum (Flesch & Jackson, 1962).

Fourthly, the time available for the histidase to act after the cell membranes become impermeable, but before the cells become too acidic for the enzyme to be active, will determine the efficiency with which the available histidine is converted into urocanic acid. This factor may be variable between species; preliminary results (not shown) indicate that in the albino rat the conversion is 95% complete, whereas published data on the histidine and urocanic acid contents of the human stratum corneum suggest that the efficiency of conversion is only approx. 30% (Pratzel & Fries, 1977).

The fact that human epidermis is so relatively inefficient at producing urocanic acid compared with other species apparently less in need of protection against u.v. light, owing to their fur, suggests that the presence of urocanic acid in the epidermis does not provide a significant evolutionary advantage, thus casting doubt on its importance as a natural sunscreening agent.

Finally, the total thickness of the stratum corneum, controlled by the balance between differentiation and desquamation, will control the urocanic acid content.

These factors are clearly not independent of each other, and therefore a given treatment may cause variation of urocanic acid concentration in a complex manner, depending on the severity and duration of the stimulus.

References

Baden, H. P. & Gavioli, L. (1974) *J. Invest. Dermatol.* **63**, 479–481

Baden, H. P. & Pathak, M. A. (1967) *J. Invest. Dermatol.* **48**, 11–17

Bailey, P. J. (1971) *Br. J. Dematol.* **85**, 264–271

Ball, R. D., Walker, G. K. & Bernstein, I. A. (1978) *J. Biol. Chem.* **253**, 5861–5868

Bergmeyer, H. U., Bernt, E. & Hess, B. (1963) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), p. 736, Academic Press, London

Brand, L. M. & Harper, A. E. (1976) *Biochemistry* **15**, 1814–1821

Eisenthal, R. & Cornish-Bowden, A. (1974) *Biochem. J.* **139**, 715–720

Everett, M. A., Anglin, J. H. & Bever, A. T. (1961) *Arch. Dermatol.* **84**, 717–719

Feigelson, M., Bhargava, M. M. & La Martiniere, C. A. (1976) in *Progress in Differentiation Research* (Muller-Berat, N., ed.), pp. 467–476, North-Holland Publishing Co., Amsterdam

Flesch, P. & Jackson, E. C. (1962) *Arch. Dermatol.* **85**, 84–92

Fukuyama, K. & Epstein, W. L. (1966) *J. Invest. Dermatol.* **47**, 551–560

Giles, K. W. & Myers, A. (1965) *Nature (London)* **206** 93

Hais, I. M. & Strych, A. (1968) *Experientia* **24**, 231–232

Hakanson, R., Ronnberg, A. L. & Sjolund, K. (1974) *Anal. Biochem.* **59**, 98–109

Klee, C. B. (1971) *Methods Enzymol.* **17B**, 69–73

Kral, J. A., Zenisek, A., Strych, Z., Hais, I. M., Petranova, O., Kalouskova, A. & Havorka, J. (1967) *Parfuem. Kosmet.* **48**, 193–195

Kral, J. A., Zenisek, A., Strych, Z. & Hais, I. M. (1968) *Cas. Lek. Cesk.* **107**, 302–307

La Du, B. N. (1972) *Metab. Basis Inherited Dis.*, 3rd edn., pp. 338–350

Magasanik, B., Kaminskas, E. & Kimhi, Y. (1971) *Methods Enzymol.* **17B**, 45–51

Marples, M. J. (1965) *The Ecology of the Human Skin*, pp. 130–135, Charles C. Thomas, Springfield, IL

Matsumura, T., Yamanaka, T., Hashizume, S., Irie, Y. & Nitta, K. (1975) *Jpn. J. Exp. Med.* **45**, 377–382

Nikolaev, A. Ya., Osipov, E. V., Burobin, V. A. & Kozlov, E. A. (1977) *Biochemistry (Engl. Transl.)* **42**, 865–869

Pratzel, H. & Fries, P. (1977) *Arch. Dermatol. Res.* **259**, 157–160

Prottey, C., Hartop, P. J. & Ferguson, T. F. M. (1972) *Br. J. Dermatol.* **87**, 586–607

Prottey, C., Tovell, P. W. A. & Ferguson, T. F. M. (1974) *Br. J. Dermatol.* **91**, 667–679

Rechler, M. M. & Tabor, H. (1971) *Methods Enzymol.* **17B**, 63–69

Tabachnik, J. (1959) *J. Invest. Dermatol.* **32**, 563–568

Vasantha, L. (1970) *Indian J. Med. Res.* **58**, 1079–1084

Wadia, A. S., Sule, S. M. & Mathur, G. P. (1975) *Indian J. Exp. Biol.* **13**, 234–237

Zannoni, V. G. & La Du, B. N. (1963) *Biochem. J.* **88**, 160–162